HHS Public Access

Author manuscript

Adv Exp Med Biol. Author manuscript; available in PMC 2018 May 17.

Published in final edited form as:

Adv Exp Med Biol. 2016 ; 892: 33–68. doi:10.1007/978-3-319-25304-6_3.

Proton Transport and pH Control in Fungi

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Abstract

Despite diverse and changing extracellular environments, fungi maintain a relatively constant cytosolic pH and numerous organelles of distinct lumenal pH. Key players in fungal pH control are V-ATPases and the P-type proton pump Pma1. These two proton pumps act in concert with a large array of other transporters and are highly regulated. The activities of Pma1 and the V-ATPaseare coordinated under some conditions, suggesting that pH in the cytosol and organelles is not controlled independently. Genomic studies, particularly in the highly tractable *S. cerevisiae*, are beginning to provide a systems-level view of pH control, including transcriptional responses to acid or alkaline ambient pH and definition of the full set of regulators required to maintain pH homeostasis. Genetically encoded pH sensors have provided new insights into localized mechanisms of pH control, as well as highlighting the dynamic nature of pH responses to the extracellular environment. Recent studies indicate that cellular pH plays a genuine signaling role that connects nutrient availability and growth rate through a number of mechanisms. Many of the pH control mechanisms found in *S. cerevisiae* are shared with other fungi, with adaptations for their individual physiological contexts. Fungi deploy certain proton transport and pH control mechanisms not shared with other eukaryotes; these regulators of cellular pH are potential antifungal targets. This re view describes current and emerging knowledge proton transport and pH control mechanisms in *S. cerevisiae* and briefly discusses how these mechanisms vary among fungi.

Keywords

S. cerevisiae; proton pump; cytosolic pH; organelle acidification; pH sensing and growth; pH signaling; V-ATPase; Pma1

1. Introduction

Tight control of cytosolic and organelle pH is critical for viability in all eukaryotic cells, including fungi. Protein conformation is exquisitely sensitive to pH. Most enzymes have a pH optimum for activity, and this property is exploited to provide pH-dependent, organelle-specific activity regulation. The pH gradient across the inner mitochondrial membrane drives ATP synthesis, and pH gradients across membranes can drive nutrient import coupled to transport of ions and other solutes. In recent years, the importance of pH as an intracellular

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signal has begun to come into focus. Given the diverse and critical functions directly linked to pH control, it is not surprising that genomic studies indicate that cytosolic pH can quantitatively control growth rate in *S. cerevisiae*.

However, many fungi encounter particularly challenging environments for pH control. They tolerate wide ranges of extracellular pH and must adapt to wide-ranging concentrations of other ions that facilitate or challenge pH control. Strains such as *S. cerevisiae* that frequently utilize aerobic glycolysis rapidly acidify their surroundings and generate copious amounts of organic acids. As a result, fungi have robust mechanisms for pH control and H^+-transport, incorporating both mechanisms common to all eukaryotes and specialized factors that facilitate adaptation to more extreme conditions. Interestingly, pH control in yeast is of considerable practical interest as well, as weak acids such as sorbate are widely used as preservatives to inhibit fungal growth. Thus, pH control in fungi can be viewed both as remarkably adaptable and as an Achilles heel.

This review outlines current knowledge of fungal proton transport and pH control, focusing initially on *S. cerevisiae*, where a wide array of genomic, biochemical and cell biological tools are available to address the proton transporters and underlying mechanisms of pH control. pH regulation in other filamentous fungi, and particularly pathogens, will then be compared to *S. cerevisiae*, and the possibilities for designing antifungal agents that target pH control mechanisms will then be addressed.

2. The physiological context of pH homeostasis in S. cerevisiae

In *S. cerevisiae*, as in other organisms, pH control mechanisms are adapted to the cells' physiological context. Under conditions of abundant glucose, their preferred carbon source, *S. cerevisiae* cells will undergo rapid fermentative growth, producing ethanol, CO_2 and organic acids through glycolysis (reviewed in[1,2]). Cells grown in glucose rapidly acidify their medium and require robust mechanisms to maintain cytosolic pH during growth, and cytosolic pH decreases as cells reach stationary phase (reviewed in [3]). Although *S. cerevisiae* is quite tolerant of ethanol, ethanol production ultimately limits growth, and this limitation may reflect a combination of plasma membrane permeabilization at high alcohol concentrations, which compromises nutrient uptake, and a resulting inability to control cytosolic pH. Interestingly, recent experiments have indicated that ethanol tolerance can be substantially increased by preventing extracellular acidification during fermentation and including excess K+ in the medium [4]. These modifications promote activity of the plasma membrane proton pump, and highlight the central importance of maintaining pH gradients and plasma membrane potential for cell viability and growth. It should be noted that under glucose-rich conditions, there is very little oxidative phosphorylation in *S. cerevisiae*, and transcription of proteins in the respiratory chain and the ATP synthase is strongly repressed [5]. Consistent with a limited role for oxidative phosphorylation as a source of ATP under fermentative conditions, respiratory inhibitors like antimycin A have little effect on cytosolic pH in cells grown in glucose [6]. *S. cerevisiae* can also grow on non-fermentable carbon sources such as glycerol and ethanol, and in fact, will shift to metabolism of ethanol as a carbon source during prolonged growth when glucose is exhausted [5]. During growth on

non-fermentable carbon sources, synthesis of the enzymes required for oxidative phosphorylation is derepressed [5], and overall growth is generally slower.

Superimposed on the requirement for cytosolic pH control is a requirement for precise control of organellar pH [7]. All cells have a number of organelles, including vacuoles/lysosomes, endosomes, and the Golgi apparatus that maintain an acidic lumenal pH relative to the cytosol (reviewed in [8,9]). The internal pH of these organelles is tuned to their functions: for example, vacuolar proteases have optimal activity at acidic pH and the affinity of various receptor-ligand complexes is tuned to compartment pH. In contrast, mitochondria are alkaline relative to the cytosol, consistent with the requirements for a membrane potential across the mitochondrial inner membrane and for a pH gradient able to drive ATP synthesis during oxidative phosphorylation [3]. Under conditions where cytosolic pH control is challenged, the impact on organelle pH must also be considered. An overview of the cellular pH gradients in cells at log phase in glucose is depicted in Fig. 1.

In virtually all cells, pH control is achieved primarily by the balanced activity of three types of molecules: pumps, exchangers, and buffers, coupled to multiple layers of regulation [9]. Proton pumps, including Pma1 at the plasma membrane and V-ATPases on organelle membranes in fungi, couple hydrolysis of ATP to proton transport and thus are key players in establishing pH gradients [3]. Exchangers can exploit the energy stored in pH gradients to transport ions and solutes against a gradient or to assist in pH control using the gradient of another ion, and may represent the source of the “proton leak” that helps to determine final pH in various organelles [10]. Buffers, particularly the robust phosphate buffering system in *S. cerevisiae* [11], protect cells and organelles from short-term pH transients, but cannot withstand long-term shifts without assistance from proton transporters [9].

3. The plasma membrane H^+-pump Pma1 and organellar V-ATPases: central players in cellular pH control

3.1 Pma1 structure, function, and genetics

Pma1 is a single-subunit P-type H^+-ATPase belonging to the same family as the ubiquitous Na^+/K^+-ATPase of mammalian cells [12]. It is the most abundant protein of the *S. cerevisiae* plasma membrane and the major determinant of plasma membrane potential, as a result of its electrogenic transport of H^+ without counterions [13]. It is believed to be the primary determinant of cytosolic pH, and is a major consumer of cellular ATP [12]. Pma1 has ten transmembrane domains, cytosolic N- and C-termini, and a large intracellular loop between the 4th and 5th transmembrane helices [14]. Aspartate 378 of *S. cerevisiae PMA1* resides in the large intracellular loop and forms the characteristic β-aspartyl-phosphate intermediate during each catalytic cycle [15,16]. Pma1 homologues are found in all fungi, as well as in plants. Although there is no high-resolution structure of any fungal Pma1, the *Neurospora* Pma1 was modeled by incorporating insights from the related SERCA Ca^{2+}-ATPase structure into an 8 A map of the *Neurospora* proton pump from electron microscopy [14]. Subsequently, an X-ray crystal structure of the related *Arabidopsis* Pma1 in complex with the non-hydrolyzable ATP analog AMP-PNP was solved in 2007 [16]. Consistent with the predictions of the model, the plant Pma1 structure revealed that, like previously

characterized P-type pumps [17–19], Pma1 folds into the three cytosolic domains, designated P for phosphorylation, N for nucleotide binding, and A for actuator, despite rather low sequence identity with the previously crystallized pumps [16]. In other P-type ATPases, ATP binding and hydrolysis drives large conformational changes between the three cytosolic domains that are communicated to the transmembrane helices to drive directional ion transport [20]. It is very likely similar mechanisms are present in Pma1. The plant Pma1 structure also provided insights into the possible path of proton transport, involving conserved amino acids in the transmembrane helices [16,21]. Although many P-type ATPases exhibit counterion transport, there is no evidence of any other ion being transported by Pma1. A model for the *S. cerevisiae* Pma1 was generated using the Phyre2 server [22]. The highest confidence model was based on the *Neurospora* model [14] (*N. crassa* Pma1 has a higher degree of sequence identity with the *S. cerevisiae* pump that the *A. thaliana* enzyme) and is shown in Fig. 2A.

Consistent with its central role in pH control and bioenergetics at the plasma membrane, *PMA1* is an essential gene in *S. cerevisiae* [12]. Mutations that partially compromise Pma1 function generally result in reduced growth rate, particularly at acidic extracellular pH, as well as resistance to hygromycin [23]. Hygromycin resistance has been attributed to depolarization of the plasma membrane as a result of loss of electrogenic proton transport through Pma1 and has been used to isolate Pma1 mutants [24,25]. Certain *pma1* mutations also result in a distinctive multi-budded phenotype [23]. The central role of Pma1 in fungal bioenergetics and its absence from higher eukaryotes also suggested that it might be a target for antifungal development [26].

S. cerevisiae has a paralog of *PMA1* that likely arose during the whole genome duplication. The open reading frame of *PMA2* is highly conserved, but *PMA2* is expressed at much lower levels than *PMA1* and has different catalytic properties [27]. It can partially compensate for the lethality of a *PMA1* deletion if overexpressed from the *PMA1* promoter [28], but even under these conditions, only low concentrations reach the plasma membrane, and phenotypes such as sensitivity to low pH are observed. Unlike *PMA1*, deletion of *PMA2* gives few phenotypes, and thus its overall physiological function is not clear. The *pma2Δ* mutant was identified as having greatly decreased filamentation in a genomic screen for genes required for filamentous growth, but the mechanistic implications of this have not been examined [29].

3.2 V-ATPases: Structure, function, and genetics

V-ATPases are multisubunit proton pumps that acidify organelles such the vacuole/lysosomes, endosomes, and Golgi apparatus of all eukaryotic cells [7,30]. These pumps are very highly conserved among eukaryotes, and consist of a complex of peripheral membrane subunits containing sites of ATP hydrolysis (the V_1 subcomplex) attached to a complex of integral membrane proteins that comprise the proton pore (the V_o subcomplex). Although there is no high resolution structure of any assembled V-ATPase, an 11 A structure from cryo-EM [31] is shown in Fig. 2B. The pump is oriented with the V_1 complex toward the cytosol to allow ATP-driven proton transport into organelles. Although V-ATPases can

reside at the cell surface of certain mammalian cells [30], they appear to be exclusively intracellular in fungi.

V-ATPases have an evolutionary relationship to F-type ATP synthases and archaeal ATPases and ATP synthases [32–34]. All of these enzymes consist of peripheral and integral membrane subcomplexes that separate the ATP-binding and proton translocation functions and communicate ATP binding state to the proton pore through long-range conformational changes. All three types of proton pumps exhibit rotary catalysis, which requires that conformational changes in the catalytic subunits with ATP binding and hydrolysis be transmitted to central rotor stalk in the center of the catalytic complex [35–37]. This central stalk is capable of turning a membrane rotor complex consisting of several protonatable proteolipid subunits. Proton transport then occurs at the interface between the proteolipid subunits and a single larger membrane subunit (V_o subunit a) [30]. Productive rotary catalysis requires not only a rotor, but also stator connections that maintain stable connections between the hexamer of catalytic and regulatory subunits and the V_o a subunit. Eukaryotic V-ATPases have the most complex stator subunit arrangement of any of the rotary proton pumps with three peripheral stator stalks [38,31], while F-type and archaebacterial ATPases have one and two respectively [39–41]. In addition, while the stator stalks of F-type and archaebacterial ATPases are tightly associated with the membrane sector [42–44], the three peripheral stalks of V-ATPases (consisting of subunits E and G) associate tightly with the peripheral V_1 sector [45,38]. Two bridging subunits, V_1 subunits H and C, provide the primary contacts between the V_1 and V_o sectors [46,47,31]. This more complex arrangement in the stator subunits may support the regulatory reversible disassembly of V-ATPases (see below). The highest levels of sequence identity between F- and V-type ATPases are found in the catalytic and regulatory ATP-binding subunits of V-ATPases, and the proteolipid subunits, while other subunits show structural similarity, but limited sequence identity [32]. In contrast, sequence identities between V-ATPase subunits of different organisms tend to be very high [30].

Higher eukaryotes have multiple isoforms for many of the V-ATPase subunits that impart both tissue- and organelle-specific regulation on V-ATPases [30]. In general, fungi show a more limited spectrum of isoforms. *S. cerevisiae* has a single set of two subunit isoforms for the largest membrane subunit. These two isoforms, Vph1 and Stv1, have a distinct cellular distribution with Vph1 residing primarily in the vacuole at steady state, and Stv1 cycling between Golgi and endosomes, with most Stv1 visualized in the Golgi [48,49]. Overexpression of *STV1* can suppress the phenotypes of a *vph1Δ* mutation and Vph1 is believed to mask the phenotype of *STV1* deletion [48]. However, V-ATPases containing Vph1 and Stv1 have distinct biochemical properties, suggesting that they are not fully functionally redundant [50].

Loss of V-ATPase activity is conditionally lethal in fungi, but lethal in higher eukaryotes [51,52]. Deletion of any of the single copy V-ATPase subunit genes (*vma* mutants) or deletion of both *STV1* and *VPH1* generates a very characteristic Vma^- growth phenotype. This phenotype is characterized by slow growth under all conditions, with optimal growth at extracellular pH 5 [51]. *vma* mutants are unable to grow at alkaline extracellular pH or under high calcium concentrations [53], and the mutants are sensitive to a wide array of heavy

metals and drugs ([54]; reviewed in [55]). *vma* mutants have proven to be very valuable in characterizing V-ATPase function *in vivo*, and the viability of loss-of-function mutants has made *S. cerevisiae* a major organism for studies of V-ATPase structure and mechanism.

3.3 Regulation of Pma1

In *S. cerevisiae*, Pma1 and V-ATPases function in a physiological context characterized by variable availability of carbon sources and wide-ranging extracellular pH. Like many cancer cells, *S. cerevisiae* grows very rapidly under conditions of aerobic glycolysis when glucose is abundant [56]. This generates large amounts of organic acid and exerts significant pH stress on cells [3]. With their exposure to the cytosol and dependence on cytosolic ATP, it is not surprising that Pma1 and the V-ATPase respond to certain common environmental factors such as glucose levels and intracellular and extracellular pH. Glucose metabolism activates both pumps, consistent with a role in removing metabolically generated protons from the cytosol. Their responses to pH are more complex, but mutations in either class of pump can generate pH-dependent lethality, consistent with their central role in cellular pH homeostasis. The functions of these two classes of pumps are increasingly recognized as interconnected, although their individual regulatory mechanisms are quite different. These distinct modes of regulation will be outlined first, followed by evidence for coordination of Pma1 and V-ATPase activity.

Reversible activation of Pma1 in response to glucose was first reported by Serrano in 1983 [57]. Incubation of yeast cells in glucose resulted in a 10-fold activation of ATPase activity that was rapidly reversed by glucose removal. Glucose activation was accompanied by a decrease in K_m for ATP and a shift toward neutral pH optimum for activity. The biochemical basis for this regulation has been a subject of debate for some time. A number of P-type ATPases are regulated by autoinhibitory interactions between their C-terminal tail and the catalytic site, with activation of the enzyme arising from relief of these interactions. Deletion analysis of the Pma1 C-terminal tail implicated this region of the yeast pump in glucose regulation, since deletion of the last 11 amino acids resulted in glucose-independent activation [58]. Mutation of two amino acids in the deleted region, S911A and T912A, abolished glucose activation of Pma1 and compromised growth of cells on glucose [59]. Significantly, a number of second site suppressors of the glucose-dependent growth defects mapped to mutations near the catalytic site [59], consistent with the proposed autoinhibitory interaction. Chang and Slayman provided convincing evidence of phosphorylation and dephosphorylation of Pma1 at the plasma membrane in response to glucose addition and deprivation, respectively [60]. This led to the hypothesis that phosphorylation of the C-terminal tail was involved in relief of autoinhibition, and attention focused on S911 and T912 of Pma1, which are highly conserved among fungi, along with another highly conserved serine S899 [61–64]. A phosphomimetic mutation at S899, S899D, partially mimicked the effects of glucose addition, decreasing the K_m for ATP even in the absence of glucose, but did not change the V_{max} in the absence of glucose [59]. Ptk2, a member of a family of fungal kinases, is required for full glucose activation of Pma1 [65], and Ptk2 was later shown to generate S899-dependent phosphorylation of a Pma1 C-terminal peptide in vivo [63]. However, phosphoproteomic analysis of the Pma1 C-terminal tail has provided the most definitive data to date that phosphorylation of S911 and T912 are the primary

mediators of glucose activation of Pma1 [64]. A Pma1 peptide containing amino acids 896–918 was isolated from differentially labeled glucose-starved and glucose-fermenting yeast cells by LC-MS. Comparison of the phosphorylation state of this peptide indicated that glucose-starved cells contained predominantly singly phosphorylated peptide on T912, while glucose-fermenting cells exhibit a major shift to a species with both S911 and T912 phosphorylated. In contrast, no phosphorylation of S899 was observed under either condition in these experiments. Taken together, these data support an autoinhibition of Pma1 under conditions of glucose starvation that occurs through interactions between a singly phosphorylated C-terminal tail and the catalytic domain. This inhibition is relieved upon glucose addition through phosphorylation of S911 [64]. It is still possible that other sites, such as S899, are involved in fine-tuning this regulation under different conditions. Consistent with a complex regulatory response to glucose, multiple protein kinases have been implicated in glucose regulation of Pma1 [65,66], and the primary kinases responsible for phosphorylation of S911 and T912 have yet to be conclusively defined.

The *Arabidopsis* H^+-ATPase structure provides limited insights into how autoinhibition might work, even though some form of direct or indirect autoinhibition involving the C-terminal tail is likely to occur in the plant proton pumps as well [66,16]. The pump was crystallized in a detergent-activated form, and most of the C-terminal tail was not resolved. Nevertheless, it was suggested that the C-terminal regulatory domain might inhibit activity by restricting movements of the A-domain [16,21]. The model for the *S. cerevisiae* enzyme in Fig. 2A, shows the C-terminus interacting with the N-domain. In fact, any interaction with the C-terminal tail that restricts inter-domain movement could provide autoinhibition.

Pma1 is also regulated by both intracellular and extracellular pH [67,68]. Depending on the composition of the growth medium, yeast cells can generate (and tolerate) an extracellular pH as low as pH 2–3 as they grow. Pma1 is activated in response to decreases in intracellular pH as long as glucose is present [69,70]. However, as glucose is depleted and organic acids such as acetic acid are produced, cytosolic pH falls from neutral or slightly alkaline in actively growing cells to approximately pH 6 in stationary phase cells [71]. The glucose requirement for Pma1 activation at low pH reflects the need for a continued supply of cytosolic ATP. However, the mechanisms of Pma1 activation in response to low cytosolic pH are not exactly the same as those seen with glucose activation. Specifically, at low cytosolic pH, the K_m for ATP decreases without the corresponding increase in V_{max} or shift in pH optimum that is seen in glucose activation of Pma1 [67].

As an electrogenic pump, Pma1 activity is also sensitive to plasma membrane potential, and this accounts for the significant interdependence of K^+ and H^+ transport [72,24]. The Trk1 and Trk2 K^+ transporters play a major role in this interdependence. *trk1Δ* mutants share phenotypes such as sensitivity to extracellular acidification with mutants that have reduced Pma1 activity, suggesting that loss of Trk1 limits Pma1 activity [73]. K^+ addition promotes H^+ efflux from yeast cells treated with glucose to activate Pma1 [74]. Yenush et al. uncovered a very interesting pH-dependent regulation of Trk1, which highlights the interdependence of these two systems [75]. Trk1 is inhibited by the Ppz1 and Ppz2 protein phosphatases, and the activity of these phosphatases is in turn inhibited by regulatory protein Hal3. Interactions between Hal3 and Ppz1 proved to be pH-dependent. Specifically, at low

cytosolic pH, Hal3 associated more strongly with Ppz1, relieving Trk1 inhibition, while at pH 7.5, Hal3 was released and Trk1 inhibition by Ppz1 was promoted. This regulation would appear to correlate well with pH-dependent regulation of Pma1, since at low cytosolic pH, Trk1 would be better able to neutralize the membrane potential generated by an activated Pma1, while at higher pH, less Trk1 activity would be necessary in the presence of a less active H^+ pump. Transport of monovalent cations is described in more detail elsewhere in this volume.

3.4 Regulation of V-ATPases

Like Pma1, V-ATPases are also regulated by both glucose and pH. Glucose regulation of V-ATPases involves the process of reversible disassembly of peripheral V_1 subunits from the membrane-bound V_o sector (reviewed in [76]). Loss of V_1-V_o association upon acute glucose deprivation in yeast was first observed by immunoprecipitation of assembled V-ATPase complexes, free (i.e. not bound to V_o) V_1 complexes, and free (i.e. not bound to V_1) V_o complexes with subunit-specific monoclonal antibodies [77]. These experiments showed that in glucose-replete cells, the majority of V_1 complexes were bound to V_o, but upon acute glucose depletion, the V_1 and V_o sectors were predominantly separated. Interestingly, one of the subunits involved in bridging V_1 and V_o, V_1 subunit C, was released from both V_1 and V_o sectors. Vacuoles isolated from glucose-deprived cells also have reduced levels of V_1 subunits and reduced ATPase activity, consistent with disassembly representing a mechanism of V-ATPase inhibition. Disassembly of V_1 from V_o is fully reversible, even in the absence of protein synthesis, and repeated cycles of V-ATPase assembly and disassembly in living cells were observed using a microfluidics format [78]. The physiological relevance of this mechanism in controlling organelle acidification is supported by the observation that vacuolar pH rises after even a brief (10–15 min.) period of glucose deprivation, but rapidly decreases upon glucose readdition [74,79]. Recent experiments with GFP-tagged proteins have suggested that many V_1 subunits may stay in proximity to the vacuolar membrane during periods of glucose deprivation, with only V_1 subunit C assuming a fully cytosolic localization [80]. It is possible that the extensive dissociation of V_1 observed by multiple other approaches [77,81,82,78] represents a disruption of a very fragile V_1-V_o association in glucose deprived cells or that V_1 subunits are retained in proximity to the membrane by some other mechanism, but it is clear that the V-ATPase is not functional under these conditions. Interestingly, both ATP hydrolysis in dissociated V_1 sectors and proton pumping through the free V_o complex are inhibited under conditions of disassembly [83,84]. Reversible disassembly was reported in the tobacco hornworm *M. sexta* and the yeast *S. cerevisiae* at about the same time [85,77]. Since then, reversible disassembly of V-ATPases has been observed in a number of higher eukaryotes, suggesting it is a general mode of V-ATPase assembly [86,87].

The mechanism of glucose-induced reversible disassembly is not well-understood. Neither glucose nor glucose-6-phosphate is required to promote assembly, since the initial steps of glycolysis can be bypassed [88]. A number of glycolytic enzymes, including aldolase and phosphofructokinase, appear to associate with the V-ATPase and would thus be positioned well for transmitting a glucose signal [89–91]. Consistent with such a role, Chan and Parra observed that loss of the phosphofructokinase subunit Pfk2 resulted in reduced reassembly

of the V-ATPase after glucose deprivation and readdition [91]. Bond and Forgac provided evidence implicating the ras-cyclic AMP pathway in V-ATPase disassembly [82]. Specifically, they observed a failure of V-ATPases to disassemble in *ira1Δ* and *ira2Δ* mutants. These mutant fails to downregulate ras signaling in glucose-poor conditions [2]. Constitutive activation of either ras or cAMP-dependent protein kinase A also suppressed disassembly of the V-ATPase. These results suggest that protein kinase A is upstream of V-ATPase assembly and serves as an activator of the V-ATPase. However, Dechant et al. have presented conflicting results indicating that protein kinase A activation is downstream of V-ATPase assembly [78]. At the level of the V-ATPase itself, no post-translational modifications in the yeast enzyme have been definitively associated with reversible disassembly, although there is evidence of phosphorylation of the V_1 C subunit in insect cells under conditions of reassembly [92,93]. The connection between V-ATPase assembly and activity and glucose levels remains an important and incompletely understood question, and recent evidence linking the V-ATPase to nutritional sensing and growth control have only increased its importance [94,78,95] (see Section 5).

V-ATPases are also regulated in response to extracellular pH. Diakov and Kane demonstrated increased V-ATPase activity when cells were grown in medium buffered to pH 7 than in cells grown in medium buffered to pH 5 [96]. The elevated ATPase activity was accompanied by higher levels of V_1 subunits associated with the membrane in isolated vacuolar membranes, suggesting that V-ATPase activation in response to pH also occurs in at the level of V_1-V_o sector assembly. Disassembly in response to glucose deprivation was largely suppressed at extracellular pH 7, consistent with a stabilization of the intact V-ATPase at high pH [97,78]. It is interesting to note that while both the V-ATPase and Pma1 can be activated in response to pH, Pma1 is activated at low extracellular pH and the V-ATPase is activated at high extracellular pH. This may have implications for the balanced activity of the two pumps as described below.

Sodium ions generally do not really play the central bioenergetic role in fungi that they do in mammalian cells. In general, they are treated as toxins by the cell and are exported across the plasma membrane or sequestered (reviewed in [98]). High extracellular concentrations of NaCl induce stress responses in yeast [99,100]. Vacuolar uptake of Na^+ ions is part of the immediate response to salt stress, and occurs by Na^+/H^+ exchange [101,102]. V-ATPases establish the required H^+ gradient and are strongly activated in response to salt stress [102,103]. This activation also occurs through increased assembly of V_1 and V_o subunits. Interestingly, the salt activation of the V-ATPase is almost completely dependent on the presence of the signaling phosphoinositide phospholipid $PI(3,5)P_2$ [104]. This lipid of the vacuole and late endosome increases up to 20-fold in response to salt stress [105], and recent data indicates that it interacts directly with the V_o sector of the V-ATPase and promotes stable V_1-V_o assembly [104]. Notably, lack of $PI(3,5)P_2$ has no effect on reversible disassembly in response to glucose, suggesting that multiple signaling mechanisms regulate the level of V-ATPase assembly and activity [104].

3.5 Coordination of V-ATPase and Pma1 activity

For many years V-ATPase and Pma1 activities were treated as largely independent. Pma1 was regarded as the major regulator of cytosolic pH, and V-ATPases were assigned an independent role in organelle acidification. Understanding the relationship between cytosolic and vacuolar pH control requires coordinated measurement of these parameters, as well as their responses to relevant regulators, in living cells. Cytosolic and vacuolar pH have been measured in whole yeast cells by a number of methods such as ^{31}P-NMR, and such measurements have suggested a coordinated response of cytosolic and vacuolar pH to weak acid stress [106,68]. However, the advent of ratiometric fluorescent pH measurements for use *in vivo* have made these measurements much more accessible. In *S. cerevisiae*, vacuolar pH can be conveniently measured with BCECF-AM, which accumulates in vacuoles in vivo [107–109]. Ratiometric measurement of pH is possible from ~pH 5–7, and normalized measurements of fluorescence intensity at 490 nm to cell density have been used to measure vacuolar pH below pH 5 [108,110]. Cytosolic pH can be measured with the ratiometric pH-sensitive GFP, pHluorin, which gives a linear response to pH from 6–8, covering the range of cytosolic pH typically seen in vivo [111,109,6]. Using these ratiometric fluorescence methods, vacuolar and cytosolic pH responses have been measured under a variety of growth conditions and in a large number of mutant strains (see below). Targeted versions of pHluorin have also been developed and used to measure Golgi and mitochondrial pH in *S. cerevisiae* [6,112].

In wild-type cells, the responses of cytosolic and vacuolar pH to glucose addition are consistent with the glucose activation of Pma1 and the V-ATPase. Cells briefly (15–30 min) deprived of glucose show a slightly elevated vacuolar pH that is reduced upon glucose addition [74,79]. The involvement of the V-ATPase in this reduction is supported by parallel measurements in *vma* mutants, which reveal that vacuolar pH is higher before glucose addition, and increased, instead of decreased, upon glucose addition[74,79]. Cytosolic pH responses measured via pHluorin were very similar to those observed previously by other methods [74,6]. In log-phase cells, cytosolic pH is decreased even by a brief glucose deprivation, and readdition of glucose results in a transient acidification followed by rapid alkalinization to neutral or slightly alkaline pH. Measurement of cytosolic pH in *vma* mutants revealed that cytosolic pH responses are significantly slower in the *vma* mutants than in wild-type cells, and cytosolic pH is somewhat lower [74,79]. This result suggested that loss of V-ATPase activity has an unexpected impact on cytosolic pH and possibly on Pma1 activity. Consistent with loss of V-ATPase activity affecting the activity of Pma1, the rate of glucose-activated proton export from cells proved to be much lower in the *vma* mutants [74,79].

A number of different mechanisms could coordinate activities of the vacuolar and plasma membrane pumps. However, Perzov et al. [113] had observed that Pma1 behaved differently in differential centrifugation and subcellular fractionation in the *vma* mutants and proposed that Pma1 was trapped in the endoplasmic reticulum of the mutants. Hirata et al. reported localization of Pma1 to the vacuole in the *vma* mutants, but the significance of this was not clear [114]. These data suggested that the levels of Pma1 at the plasma membrane might be reduced in the *vma* mutants. Immunofluorescence microscopy confirmed that Pma1 was

partially localized to intracellular compartments including the interior of the vacuole in *vma* mutants, particularly in strains that were also deficient for vacuolar proteases, suggesting that Pma1 might be targeted to the vacuole for degradation [115]. Huang and Chang provided evidence that a number of plasma membrane transporters, including Pma1, might be mislocalized to the vacuole as a result of loss of organelle acidification in the secretory pathway [116]. This is very likely to be one mechanism of coordination under conditions of long-term loss of V-ATPase activity. However, Smardon et al. demonstrated that acute loss of V-ATPase activity also results in a rapid ubiquitination and endocytosis of Pma1 from the plasma membrane in wild-type cells [117]. This endocytosis requires the ubiquitin ligase Rsp5, homologue of the mammalian NEDD4, and an adaptor protein Rim8. Importantly, double mutants containing both a V-ATPase deletion and a loss of function mutation in *RSP5* or *RIM8* grow very poorly. Furthermore, double mutants containing both a V-ATPase deletion and a mutation that prevents endocytosis are inviable [118], and treatment of an endocytosis mutant with the V-ATPase inhibitor concanamycin A resulted in rapid loss of cell integrity [117]. These data indicate that the rapid endocytosis of Pma1 upon loss of V-ATPase function likely represents a compensatory response rather than an error in trafficking. It is possible that balancing levels of Pma1 and V-ATPase activity is important for maintenance of overall pH homeostasis.

Further support for coordination of Pma1 and V-ATPase levels was recently obtained through studies of aging cells. Pma1 is an exceptionally long-lived protein that appears to be retained predominantly by mother cells during yeast cell division [119]. As a result of this, Pma1 progressively accumulates on the surface of aging cells, but there is no evidence of any parallel increase in V-ATPase levels. Loss of vacuolar acidification as yeast cells age had been reported and appeared to be associated with loss of mitochondrial function [120]. Henderson et al. [121] tested whether reducing Pma1 activity via a *pma1-105* mutation would restore vacuolar acidification in aged cells and “rejuvenate” these cells. They found that vacuolar acidification, as indicated by increased quinacrine uptake, was restored in the *pma1-105* mutant. Furthermore, they provided evidence of higher cytosolic pH in cortical regions of wild-type mother cells containing excess Pma1 than in daughter cells containing limited Pma1, suggesting that excess Pma1 in mothers alkalinizes the cytosol [121]. Taken together, these data highlight the importance of balancing the activities of Pma1 and the V-ATPase, and indicate that failure to maintain this balance has functional implications for overall pH homeostasis and lifespan [121]. One question that remains is why the Rim8/Rsp5 pathway for internalization of Pma1 is not activated in aging cells as they begin to lose the Pma1/V-ATPase balance. In addition, the signal to Rim8/Rsp5 for loss of V-ATPase activity, which might be compromised in aging cells, is not known. Further experiments are needed to address these questions.

3.6 Other regulators of cellular pH in yeast

Although the V-ATPase and Pma1 may be regarded as primary drivers of cellular pH gradients, they are not the sole regulators. Several other transporters have been implicated in control of cytosolic and/or organelle pH, and additional players are emerging from genomic screens (see below). In this section, a few of the better characterized players in pH control will be described.

In mammalian cells, Na^+/H^+ exchangers (NHE1,NHE2, and NHE3 proteins) act as the primary exporters of H^+, using the Na^+ gradient established by the Na^+/K^+-ATPase [9]. There are also $Na^+(K^+)/H^+$ exchangers in organelles (NHE6-9) that are implicated in organelle pH control [10]. Yeast cells have homologues of both classes of exchanger, which exhibit both functional similarities and differences from their mammalian homologues [122,123]. The yeast Nhx1 protein resides in late endosomes/pre-vacuolar compartments and bears homology to the intracellular exchangers [124]. These intracellular exchangers are likely to transport both Na^+ and the more abundant cytosolic K^+ [111], and thus have the potential to modulate pH by coupling export of H^+ from organelles to uptake of Na^+ or K^+ into organelles. Consistent with such a role, yeast *nhx1Δ* mutants were shown to have a more acidic vacuolar pH than wild-type cells when subjected to acid stress [108,123], but also have a more acidic cytosol, for reasons than are less clear [111]. Interestingly, Nhx1 effects on organelle pH seem to be tied to intracellular trafficking, supporting the idea that lumenal pH is sensed as part of organelle identity [111]. Plasma membrane exchangers play a key role in alkali metal tolerance, as they use the H^+ gradient across the plasma membrane to support export of Na^+ [122]. Under some circumstances, they have also been shown to influence cellular pH [125].

4. Genomic perspectives on H^+-transport and pH control

4.1 pH measurements across deletion mutant arrays

The development of ordered *S. cerevisiae* deletion mutant arrays [126] has allowed a system-level understanding of pH control that was not previously possible. Vacuolar and cytosolic pH have been compared across thousands of individual deletion strains under a defined set of conditions (medium composition, extracellular pH, and growth phase) and deviations in pH from the wild type strain have implicated sets of genes and processes in maintaining pH [71,110]. In addition, arrays of deletion strains have been tested for sensitivity to pH challenges such as weak acid stress ([69]; reviewed in [127]) and alkaline extracellular pH [128]. This type of experiment has provided insights into the requirements for survival under conditions of varied pH and the cellular functions most sensitive to pH change. A major strength of these approaches is that they have identified novel intersections between pH control and other processes, as well as new players that would never have been tested by more targeted approaches. One potential weakness is that cellular pH is a very dynamic parameter that is responsive to multiple environmental factors ranging from growth phase of the cells to pH and buffering of the medium [71,96]; as a result, conditions for screens must be carefully controlled, and the results cannot be automatically extrapolated to other growth conditions. Nevertheless, such screens have provided a wealth of new information and new candidates for pH control and pH responses.

4.2 A systems-level view of cytosolic pH control

Orij et al. introduced a cytosolic pHluorin into the deletion mutant array in order to identify critical determinants of cytosolic pH control [71]. Remarkably, in their screen of more that 4200 deletion mutants they determined that no single mutation had a measured cytosolic pH more than 0.3 pH units lower or 0.5 pH units higher than the wild-type average. The screen was conducted in defined monosodium glutamate medium buffered to pH 5 with sodium

citrate, and care was taken to ensure that the cultures were well-aerated in fresh medium and maintained in log phase growth. These parameters are critical, as they determined that lack of aeration and conditioning of the medium, presumably as a result of production of dissolved CO_2 during metabolism, had significant effects on cytosolic pH. The 73 mutants identified as having low cytosolic pH and the 104 identified as having high cytosolic pH were enriched for mutants in vacuolar proteins, including the V-ATPase, and for mitochondrial functions. The strong enrichment for mitochondrial mutants was surprising, given that pH was measured under glucose fermenting conditions, where respiration has little or no contribution to ATP generation. In addition, several categories such as ribosomal proteins and aminoacyl tRNA ligases were significantly enriched in the mutants with altered pH, even though these mutations appear to affect very general cellular functions associated with growth rate. One of the most interesting associations uncovered in this screen was a very tight correlation between cytosolic pH and growth rate [6,71]. The hypothesis that cytosolic pH controls growth rate was tested in a mutant with lower Pma1 activity where cytosolic pH could be readily adjusted; in this strain, growth rate followed cytosolic pH closely [71]. Further analysis revealed a small set of mutations that appear to uncouple cytosolic pH from growth rate. These mutants define proteins with a potential pH signaling role and include *kcs1Δ* and *plc1Δ*, two mutants implicated in inositol pyrophosphate metabolism [129]. These mutants had not previously been linked specifically with pH control, and thus represent an excellent example of the power of such genomic screens to reveal new candidates.

4.3 A systems-level view of vacuolar pH control

Brett et al. surveyed 4600 strains of the deletion mutant array for loss of vacuolar pH control using the pH-sensitive probe BCECF-AM. In their screen, cells were grown for 19 hrs. in APG medium, a synthetic medium containing arginine, phosphate, and a variety of salts [110]. It should be noted that these conditions of screening are very different than those used for the genomic cytosolic pH screen described above, which limits comparison of the two screens. The authors screened the deletion collection at extracellular pH 2.7, 4, and 7, allowing an assessment of the response of vacuolar pH to altered extracellular pH. Under these conditions, the median vacuolar pH for wild-type cells was the same at pH 2.7 and 4 (pH= 5.27–5.28), but increased (to pH 5.83) in medium buffered pH 7. 107 mutants displayed acidic or alkaline vacuoles relative to wild-type cells in more than one external pH condition. This collection of mutants was significantly enriched in functional categories of transporters, membrane organization and biogenesis, and membrane trafficking. As expected, the V-ATPase mutants were prominent among strains with vacuolar alkalinization, and there is evidence that several of the other mutants, including mutants affecting ergosterol biosynthesis [130], likely alter vacuolar pH by virtue of effects on the V-ATPase. Intriguing patterns of vacuolar pH perturbation emerged among the trafficking mutants, with mutations implicated in defective retrograde trafficking frequently resulting in a more alkaline vacuolar pH and mutations in anterograde trafficking pathways generating vacuolar acidification [110]. This trend deserves further exploration, as it may indicate that regulated transport of specific pH regulators is a critical determinant of organelle pH. No correlation between growth rate and vacuolar pH control was observed in this genomic screen, suggesting that

cytosolic pH may be a dominant factor in determining overall growth. However, the very different conditions in the two screens may make such conclusions premature.

4.4 Cellular responses to pH stress

Whole genome transcriptional studies of cellular responses to stress, including pH stress, were reported within a few years of the completion of the *S. cerevisiae* genome. Causton et al. examined the acute transcriptional response upon shifting cells from pH 6 to acidic (pH 4) or alkaline (pH 7.9) conditions [131]. They observed significant overlap between genes induced by these stresses and by other apparently unrelated stresses such as heat shock, peroxide, and osmotic stress, and characterized this set of genes as a "common environmental response" to environmental challenges. Many of these genes, including several induced by acid or alkali were targets of the Msn2 and Msn4 transcription factors [131]. In this study, only a handful of genes showed inverse responses to acid and alkali that might be indicative of pH-responsive genes. These genes included *PDR12*, which is induced under acid conditions and repressed under alkali; *PDR12* encodes an ABC transporter important for weak acid resistance [131]. The potassium transporter *TRK2*, a paralogue of *TRK1*, showed a similar pattern of expression. In contrast, *PHO89*, encoding the major phosphate importer active at alkaline pH [132], was upregulated at alkaline pH and repressed upon a shift to low pH [131]. This control was later shown to involve activation of the calcineurin-dependent transcription factor Crz1p and repressors regulated by the Rim pathway and Snf1p [132].

Subsequent experiments, often combining microarray analysis of transcriptional responses with phenotypic screening for growth of the deletion mutant array under high or low extracellular pH, have expanded understanding of the transcriptional networks activated at high and low pH. In addition, screening of the deletion mutant array has helped to identify participants in pH responses that are not regulated at the level of transcription.

There have been multiple genomic screens for sensitivity to different weak acids (reviewed in [127]). These screens are motivated by the commercial and therapeutic importance of permeant weak acids, as well as their prominent role in normal fungal metabolism. Permeant weak acids are commercially important as fungal growth inhibitors [133]. Several weak acids are also used therapeutically, but their "off-target" cellular impacts are not fully understood [127]. In addition, fungi naturally produce organic acids during fermentative growth [134]. Weak acids are usually uncharged at low extracellular pH and can cross the membrane and dissociate in the higher pH of the cytosol. This is stressful to the cells, as dissociation of the acid generates both protons, potentially reducing intracellular pH, and a counterion. Pma1 is activated by reduced cytosolic pH (Section 3.3), but activation of Pma1 into a futile cycle, where protonated weak acids re-enter immediately after proton export, can deplete cellular energy stores [69]. In addition, the counterion can also be toxic to the cells [69], and different counterions may require distinct responses. Mira et al [127] have reviewed genomic studies addressing mechanisms of adaptation to a number of different weak acids in *S. cerevisiae.* Interestingly, there appear to be both general responses to weak acid stress and specific responses to individual acids, correlated with the structure and hydrophobicity of the counterion. Msn2 and Msn4 mediate a general environmental stress

response, including a response to multiple weak acids [131,135]; their targets include chaperones and proteins involved in energy metabolism and ergosterol biosynthesis. In contrast, the transcription factor War1 responds more specifically to sorbate, and is required for upregulation of *PDR12*, a plasma membrane transporter implicated in sorbate export [136].

Screens for mutants hypersensitive to multiple weak acids have also identified proteins that respond either generally or specifically to weak acid stress. For example, multiple experiments indicate that Pma1, the V-ATPase and ergosterol biosynthesis play a central role in resistance against weak acid stress [127,69]. However, beyond these few common players, the mutants showing hypersensitivity even to closely related weak acids such as acetic and propionic acid vary significantly [127]. Thus, cells seem to detect and respond to both general features of weak acid stress and specific features of the acid's structure or cellular impact. In addition, the response to weak acid stress appears to depend on both transcriptional activation and activation of existing transporters such as Pma1.

Alkaline pH is also stressful to *S. cerevisiae*. The pH gradient across the plasma membrane is lost when extracellular pH approaches the cytosolic pH, and nutrient and ion uptake can be disrupted. Lamb et al. [137] compared transcripts at pH 4 and 8 and identified multiple alkaline-inducible genes implicated in phosphate, copper, and iron uptake, as well as cell wall and membrane maintenance. The *ENA1* gene, encoding a Na^+-ATPase, is induced alkaline conditions [138,137] and encodes a pump capable of exporting toxic Na^+ in the absence of a H^+ gradient [139]. Subsequent work has highlighted the importance of the Rim101 and Crz1 transcription factors in the transcriptional response to alkaline stress [140] and implicated the conserved Rim pathway, the Ca^{2+}/calmodulin-dependent protein phosphatase calcineurin, and Snf1 kinase in transmitting alkaline pH signals to the transcriptional apparatus [141].

The Rim pathway is quite conserved among fungi and is critical for virulence of pathogenic fungi (see below). A working model for the pathway is depicted in Figure 3. In *S. cerevisiae*, the Rim pathway involves plasma membrane pH sensor proteins, Dfg16 and Rim21, plasma membrane protein Rim9, and the alpha arrestin Rim8 that acts downstream of the plasma membrane sensors. Activation of this upstream "sensing module" of the Rim pathway appears to signal to Rim20, which permits activation of the Rim13 protease and cleavage of Rim101. Rim101 is a transcriptional repressor that represses Nrg1 transcription [142]. Nrg1, in turn represses several alkaline response genes, including *ENA1*, and this repression is relieved by the action of Rim101 on Nrg1. In addition, multiple interactions have been reported between Rim proteins, particularly Rim20 and Rim8, and the proteins of the ESCRT pathway, which are involved in formation of the multivesicular body [143,144]. Importantly, deletion of ESCRT proteins prevents proteolytic activation of Rim101 [144]. The Rim20 protein is recruited from the cytosol to endosomes by alkaline extracellular pH, and this recruitment is dependent on the ESCRT proteins as well as proteins upstream of Rim20 in the Rim pathway (Rim21, Rim8, and Rim9) [145]. These data strongly suggest that endosome metabolism is connected to pH signaling [145]. However, recent data has suggested that the ESCRT proteins can be recruited to the plasma membrane in response to alkaline pH and that Rim pathway activation could occur at the plasma membrane [146].

The site of Rim101 activation is one of several questions that are not yet clear with regard to the Rim pathway. Rim8 can be ubiquitinated [143], but it is not clear whether Rim8 ubiquitination, or ubiquitination of the plasma membrane pH sensors, is involved in signaling to Rim101 [143,147,146]. Surprisingly, there is also evidence that the Rim pathway can pay a role in the response to acid stress [148]. These issues are interesting in the larger context of pH sensing and because of the physiological importance of the Rim pathway in other fungi. As described in Section 3.5, Rim8 also is required to promote Pma1 endocytosis in response to loss of V-ATPase activity [117]. However, Pma1 endocytosis does not appear to require other Rim pathway components, suggesting that Rim8 is a multifunctional protein (A. Smardon and P. Kane, unpublished).

Screening of a deletion mutant array for reduced growth at alkaline extracellular pH identified over 100 hypersensitive mutants [128]. As expected, mutants lacking the V-ATPase were hypersensitive, but a number of mutants compromised in iron and copper homeostasis were also hypersensitive. Only the high affinity copper transporter *CTR1* and a low affinity iron/copper/zinc transporter (*FET4*) were found to increase alkaline tolerance when expressed from multicopy plasmids [128]. These results suggested that iron and copper limitation may be a primary cause of alkaline pH sensitivity, although other ions such as Ca^{2+} are certainly involved in the alkaline pH response [140] and multisubunit proteins cannot be overexpressed by this approach. Notably, certain aspects of the transcriptional response to alkaline pH also parallel the cellular response to low glucose [149]. Taken together, these results highlight that alkaline extracellular pH generate deficiencies in multiple nutrients. Interestingly, mutations in the Rim pathway components were not identified in this screen [128], which was conducted at pH 7.2 and 7.5, and thus assessed the requirements for growth under mild alkaline stress.

5. pH as a growth signal in *S. cerevisiae*

Cytosolic pH is tightly connected to the metabolic status of the cell, and thus is positioned to serve a signaling role. Although the genomic studies described above indicate that *S. cerevisiae* strains maintain remarkably similar cytosolic pH values under comparable growth conditions, it is also clear that both cytosolic and vacuolar pH can vary significantly under different growth conditions, and that pH changes with growth as nutrients are exhausted [71]. Correlations between cytosolic pH and growth rate have been observed for some time, with acidification of the cytosol generally correlated with slow growth and more alkaline cytosolic pH associated with rapid growth. Elevation of intracellular pH through expression of Pma1 in cultured fibroblasts was even shown to impart tumorigenic properties on these cells [150], leading to a hypothesis that elevated cytosolic pH promotes uncontrolled growth. Observation of synchronized *S. cerevisiae* cultures by ^{31}P-NMR suggested that there was cytosolic alkalinization as the cells crossed into G1 [151], linking cytosolic pH to cell cycle progression. Recently, several studies have begun to provide a mechanistic basis for observations connecting cytosolic pH and cell growth.

Young et al. described a mechanism for coordination of membrane biosynthesis by cytosolic pH [152]. Acidic cytosolic pH, generated by multiple methods, triggered release of the Opi1 transcriptional repressor from its binding to phosphatidic acid in the ER membrane and

permitted its transit into the nucleus. Nuclear Opi1 is able to repress transcription of multiple phospholipid biosynthetic genes [153], thus limiting membrane biosynthesis under conditions of low cytosolic pH. These experiments [152] provided new insights into how cells might coordinate synthesis of membrane precursors with nutrient availability and growth rate. One very interesting aspect of this work was the finding that cytosolic pH changes as small as 0.3 pH units (from pH 7.1 type to 6.8) were sufficient to generate large-scale release of Opi1 and large changes in transcription. This work makes it clear that pH signaling can occur even in the face of the relatively stable cytosolic pH observed in wild type cells.

Dechant et al. have provided further evidence of pH signaling as a central component of nutrient sensing and growth control [78,95]. Consistent with the results of Orij et al. [71], Dechant et al. found that growth rate and cell size correlate with cytosolic pH, with reduced glucose associated with low cytosolic pH, slower growth, and smaller cell size [95]. Depletion of Pma1 from daughter cells appeared to result in cell cycle arrest in early G1. This arrest could be reversed by increased extracellular pH, suggesting that the G1 arrest arose from cytosolic acidification. Cytosolic acidification also resulted in inactivation of Ras, consistent with low cytosolic pH acting as a signal for glucose limitation. Interestingly, this work and previous work from the same lab indicate that the V-ATPase plays a central role in this mode of pH signaling. Consistent with the results of Diakov and Kane, Dechant et al. found that V-ATPase disassembly upon glucose deprivation was suppressed at high pH, suggesting that cytosolic pH helps to signal V-ATPase assembly [96,78]. They proposed that glucose levels are sensed, at least in part, through maintenance of neutral or slightly alkaline cytosolic pH, and suggested that the assembled V-ATPase is required to transmit the pH signal to downstream effectors such as protein kinase A and Ras [78,95]. The small GTPase Arf1 was shown to interact with the Stv1-containing (Golgi/endosome) form of the yeast V-ATPase, and mutational studies placed the V-ATPase upstream of Arf1 and Arf1 upstream of Ras [95]. One puzzling aspect of the Stv1-Arf1 interaction is that Stv1-containing V-ATPases in the Golgi and endosome do not appear to undergo much glucose-induced reversible disassembly [154], suggesting that increased assembly in response to elevated cytosolic pH is unlikely to signal through Stv1-containing complexes. However, although the picture is not yet complete, these data place pH control, and the V-ATPase and Pma1 specifically, at the center of glucose signaling, with Pma1 controlling cytosolic pH, and the V-ATPase acting as a signaling intermediate between glucose levels, cytosolic pH and the critical nutrient sensors PKA and Ras. Interestingly, the V-ATPase has also been implicated in growth control through sensing of amino acids in the TORC1 pathway [94]. This pathway requires interactions between the Vph1-containing V-ATPase at the vacuole, the GTPase exchange protein Ragulator, and the small GTPase Gtr1p (a yeast Rag protein) [94,155,95]. Cytosolic pH has not been specifically implicated in this pathway, but amino acid transporters, many of which require the V-ATPase-generated proton gradient for activity, are involved, suggesting a role for vacuolar pH as well as the V-ATPase itself [156,157].

6. Proton transport and pH control in other fungi

Fungi other than *S. cerevisiae* encode homologues of many of the same transporters and regulators involved in pH control and response to pH stress, but may deploy these molecules

in different ways depending on their metabolic demands and the pH challenges encountered in their environment. Additional pH regulators are also encoded in specific fungi to complement the core pH regulatory machinery and to provide additional defenses against specific environmental challenges. A complete comparison of proton transport and pH control between fungi is far beyond the scope of this review, but we will provide a brief comparison of several fungi with *S. cerevisiae*, and discuss prospects for targeting pH homeostasis in the development of therapeutic antifungal agents.

6.1 V-ATPase and Pma1 in Neurospora crassa

Some of the earliest information about structure and function of the fungal V-ATPase and Pma1 were obtained in the non-pathogenic, filamentous fungus *Neurospora crassa*. The first fungal V-ATPase subunit genes were cloned from *N. crassa* [158,159], and much of the early biochemical characterization of V-ATPases was also done in this fungus, including electron microscopic studies that supported the resemblance to the ATP synthase [160]. The sensitivity of fungal V-ATPases to bafilomycins and concanamycins, highly specfic inhibitors that have proven to be essential tools, was established in *N. crassa*, and the inhibitor binding site was elucidated in this organism [161–164]. A number of structural studies of Pma1 were also initiated in *N. crassa*, and the model combining electron microscopic maps with information from the high resolution structures Ca2+ pumps [165,14] provided fundamental mechanistic insights and remains one of the most complete models of a fungal Pma1.

In addition, work in *N. crassa* provided important insights into the cellular roles of V-ATPase in filamentous fungi (reviewed in [166]). Both inhibition of the V-ATPase by concanamycin and genetic disruption of the catalytic subunit resulted in gross morphological alterations, as well as loss of growth at neutral pH [167,168]. When V-ATPase activity was compromised, very short and highly branched hyphae were produced. Interestingly, when mutants resistant to concanamycin were isolated, their mutations mapped not to V-ATPase genes but instead to the *pma-1* gene [168]. This provided the first genetic evidence for crosstalk between the vacuolar and plasma membrane proton pumps. Insights into the role of V-ATPases obtained in *Neurospora* helped to set the stage for exploring V-ATPases as a therapeutic target in pathogenic filamentous fungi.

6.2 pH control in pathogenic fungi

Outside their hosts, fungal pathogens such as *Candida albicans* show a preference for abundant glucose and acidic pH, much like *S. cerevisiae*. However, within human hosts, the pathogens must tolerate neutral to slightly alkaline conditions in the bloodstream and frequent cycles of glucose deprivation. In other niches such as the oral and genital tracts, varied pH and other rapidly changing environmental conditions are also present. Fungi must adapt to varied host conditions to maintain virulence (reviewed in [169]). *C. albicans* undergoes pronounced morphological changes in response to extracellular pH, with a yeast form predominating at low extracellular pH and a filamentous morphology emerging at higher extracellular pH. The ability to form hyphae appears to be critical for virulence (reviewed in [170]).

C. albicans has a plasma membrane proton pump and V-ATPase that play a central role in pH homeostasis and are very similar to the *S. cerevisiae* pumps [171–174]. Both proton pumps are critical for growth and virulence [175,172,176,173,174], and have been explored as anti-fungal targets [175,170,177]. Pma1 activity is upregulated in preparation for filamentous growth [171], and loss of cytosolic pH control is associated with loss of filamentation [178]. Similar to the phenotypes of V-ATPase mutants in *N. crassa, C. albicans* V-ATPase mutants fail to grow at high pH and are defective in hyphae formation [172]. Interestingly, the azole drugs currently in pharmacological use as antifungals may act by inhibition of V-ATPase activity [130]. These drugs target the fungal sterol ergosterol, and depletion of ergosterol reduces V-ATPase activity [130]. Future development of new antifungals targeting V-ATPases will depend on either identifying features of the V-ATPase that distinguish them from mammalian V-ATPases or targeting regulators, like ergosterol, that are not found in humans. Although V-ATPases are very highly conserved, inhibitors that distinguish between mammalian and fungal V-ATPases have been described [179,170], suggesting that development of antifungals that directly target the V-ATPase is possible.

Many pathogenic fungi rely on pathways analogous to the Rim pathway of *S. cerevisiae* for adaptation to extracellular pH [180,181,169]. Compromising these pathways can reduce virulence, so they are being actively explored as antifungal targets (reviewed in [182]). The Pal pathway in *Aspergillus nidulans* has been extensively characterized [183] and shares many features with the *S. cerevisiae* Rim pathway. PalH and PalI serve as plasma membrane pH sensors, PalF shows homology to the alpha-arrestin Rim8, PalA (orthologue of Rim20) interacts with ESCRT proteins, and PacC (orthologue of Rim101) serves as the final transcriptional regulator of the pathway (reviewed in [180,181]). The transcriptional activities of PacC in *Aspergillus* have also been well-studied and indicate a more complex regulation than that reported for Rim101 in *S. cerevisiae*, including an involvement in both acidic and alkaline pH responses [184]. In *C. albicans*, Rim101 is required for virulence, but some aspects of Rim101 function can be partially bypassed by overexpression of Rim101 targets important for cell wall structure [185]. In addition, *C. albicans* Rim101 upregulates genes required for iron acquisition at alkaline pH [186], suggesting that iron limitation is a consequence of high ambient pH in both pathogenic and non-pathogenic fungi.

Calcineurin is also an important contributor to growth of pathogenic fungi at high ambient pH, where it can act in parallel with the Rim pathway [187]. Deletion of the calcineurin catalytic subunit gene in *C. albicans* led to growth defects at alkaline pH, although it did not affect filamentation [188]. Calcineurin is also required for *C. albicans* virulence, at least in certain host niches (reviewed in [189]). Calcineurin inhibitors have been used for severe systemic fungal infections [189], but it is not clear whether the effectiveness of these inhibitors is specifically due to the effects on ambient pH responses.

Taken together, the evidence cited above indicates that there are both similarities and differences between *S. cerevisiae*, non-pathogenic fungi like *N. crassa*, and pathogenic fungi such as *C. albicans* and *Aspergillus*. The core proton transport machinery, including the V-ATPase and plasma membrane proton pump are very similar. Certain phenotypes that accompany loss of function, such as failure to grow at alkaline pH in V-ATPase mutants, are also similar, suggesting parallel physiological roles. Because of these similarities, *S.*

cerevisiae continues to be a valuable model for understanding the proton pumps, their regulation, and the consequences of their inhibition. However, it is also clear that there are aspects of pH regulation in filamentous, and particularly pathogenic, fungi that are not captured in studies of *S. cerevisiae*. These include the connections between dimorphic growth and pH regulation, which cannot be fully recapitulated in *S. cerevisiae*. Furthermore, the adaptation of pathogenic fungi to different environmental niches in their hosts can include an adjustment to ambient pH, but likely also involves sensing of other factors. Given these differences in fungal physiology and lifestyle, it is important that studies of pH transport and regulation continue to be extended to multiple fungi.

7. Conclusions and future directions

pH control is a critical requirement for growth of all organisms. Fungi have adopted a number of common strategies to address the challenges of maintaining pH control in the face of rapid metabolism and a changing extracellular environment. These strategies include a central role for organelle and plasma membrane proton pumps, with increasing evidence suggesting that the function of these two different types of pumps is actively coordinated in order to maintain overall pH homeostasis. Structural studies of these conserved pumps have provided significant insights into their mechanisms, but there is still no high resolution structure of any fungal Pma1 or assembled V-ATPase. Such structures promise to provide new mechanistic insights. These pumps tend to be adapted to optimal activity at low pH and high glucose, but multiple layers of regulation allow them to adjust activity in response to changing environmental conditions. Despite this capacity for regulation, extracellular acidification (particularly in the presence of permeant weak acids) and alkalinization of the extracellular environment are significant stresses. The response to these stresses invokes a combination of transcriptional activation to generate additional adaptive capacity and post-translational activation of constitutively expressed proteins that is still being deciphered. Beyond pH-specific stress responses, recent work has highlighted the importance of cellular pH as a potential cellular signal, capable of determining overall growth rate and driving cells into “life or death” decisions. Despite extensive work, the key question of the molecular basis of cellular pH sensing in these pathways is not understood.

The advent of whole genome approaches has greatly advanced the field of pH homeostasis and allowed identification of new players in regulation. Combination of *S. cerevisiae* deletion mutant arrays with readily introduced or genetically encoded fluorescent pH sensors has already proven to be exceptionally valuable. Some systemwide approaches, such as proteomics, have barely been applied to studies of pH homeostasis, and also promise to provide new insights into signaling pathways important for adjustment to ambient pH. In summary, the toolbox for addressing mechanisms of proton transport and pH control has rapidly expanded in recent years, but there is still much to learn before we have a true systems-level understanding of fungal pH homeostasis. The previous fruitfulness of the *S. cerevisiae* model system and the established importance of pH control in fungal pathogens will help to drive these studies forward.

Acknowledgments

Work in the Kane lab is funded by NIH R01 GM50322. Many thanks to the investigators who provided the decades of work leading to our current understanding of fungal pH transport and pH homeostasis and apologies to all whose work I was unable to cite.

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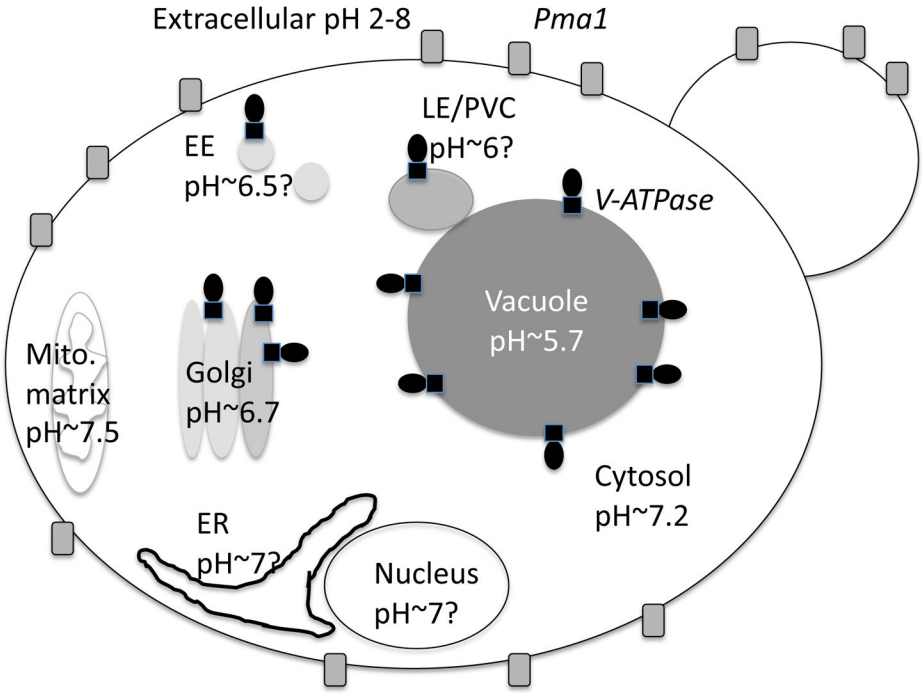

Figure 1. Compartment pH and pH gradients in glucose-grown *S. cerevisiae*

Approximate pH values for various yeast organelles are shown. Organelles are abbreviated as follows: EE, early endosome; LE/PVC, late endosome/prevacuolar compartment; ER, endoplasmic reticulum; Mito., mitochondrion. Approximate pH values that have been measured in log-phase cells growing in glucose ([111,74,6,112,79]) are shown; for compartments where organelle-specific pH measurements have not yet been made, expected pH values are indicated with a question mark. The V-ATPase is shown in black and Pma1 as grey rectangles. It should be noted that although cytosolic pH is quite constant, organelle pH is quite sensitive to growth conditions and thus will vary with buffer conditions and medium content as described in the text.

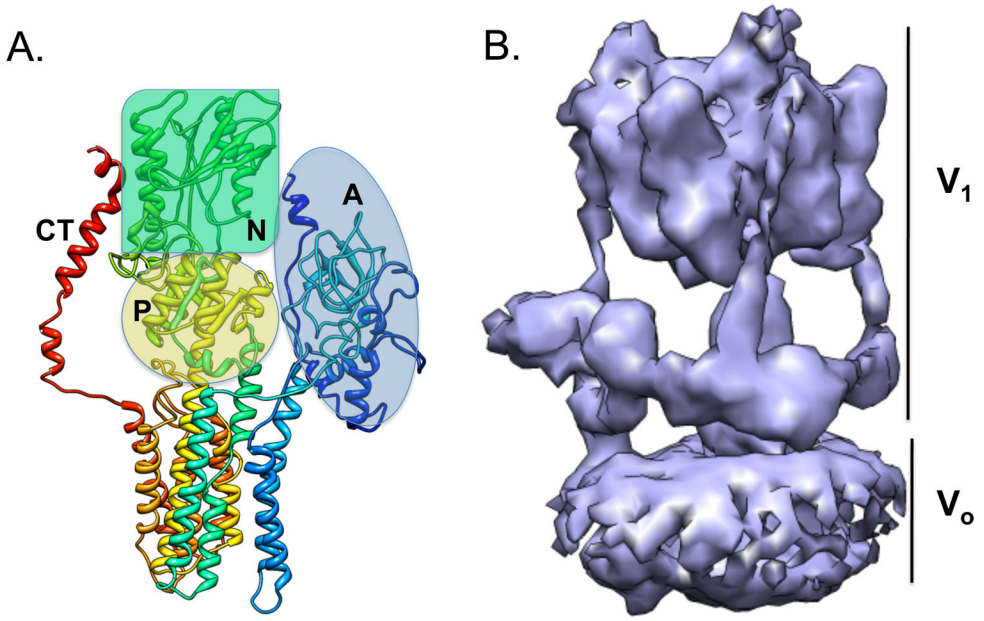

Figure 2. Structural views of the yeast Pma1 and V-ATPase proton pumps

A. The sequence of *S. cerevisiae PMA1* was submitted to the PHYRE2 server [190] for modeling. The highest confidence model is shown and was based on the *Neurospora crassa* plasma membrane H^+-ATPase (PDB 1MHS). The model is visualized using UCSF Chimera [191], and the approximate locations of the cytosolic actuator (A), nucleotide-binding (N), and phosphorylation (P) domains are indicated based on comparison to the *A. thaliana* plasma membrane H^+-ATPase structure [16]. The regulatory C-terminal tail is also indicated. B. The 11A structure of the *S. cerevisiae* V-ATPase [31] (EMDB 5476) was visualized using UCSF Chimera. The cytosolic V_1 and membrane V_o domains are indicated.

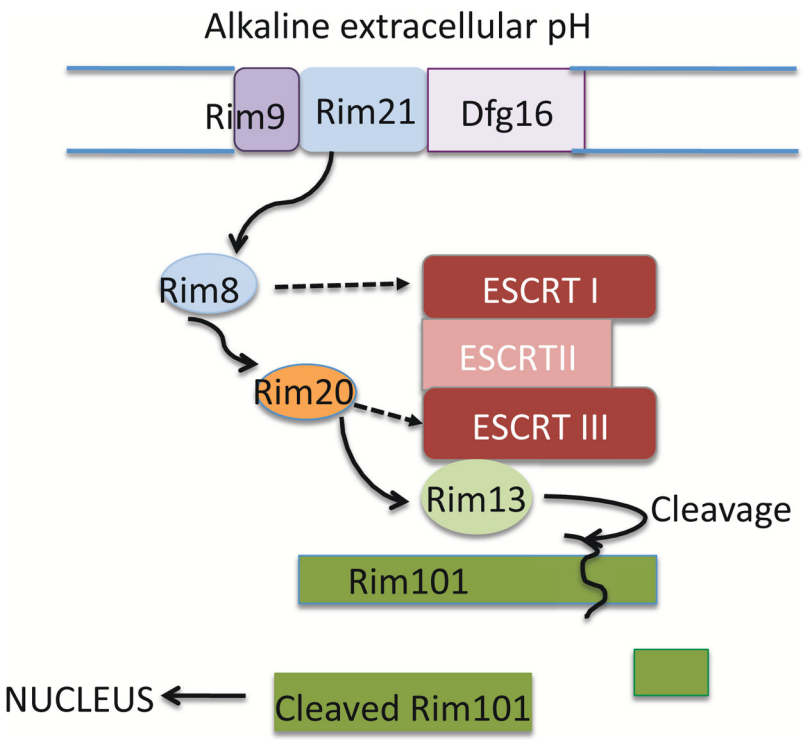

Figure 3. Working model for the *S. cerevisiae* Rim alkaline response pathway

Rim21, Dfg16, and Rim9 are plasma membrane proteins that are implicated in the initial step of ambient pH sensing. Rim8 and Rim20 represent subsequent steps in the pathway, and both have interactions with proteins of the ESCRT complex that are required for signaling. In the final steps, the Rim13 protease is activated and cleaves Rim101, which is then transported to the nucleus. Further details are provided in the text. The diagram is adapted from [182].